Identification of Platelet-Activating Factor Acetylhydrolase II in Human Skin

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Platelet-activating factor acetylhydrolases are a family of specialized phospholipase A2 enzymes. They serve an anti-inflammatory function by converting the proinflammatory autocoid, PAF, into biologically inactive lyso-PAF, by the removal of the *sn-2* acetyl group of this glycerophospholipid. Similarly, platelet-activating factor acetylhydrolases can also degrade oxidatively modified *sn-2* polyunsaturated-fatty-acid-containing phospholipids, which are toxic to cells. Platelet-activating factor acetylhydrolase II is a recently cloned member of this family of specialized phospholipases. Consistent with a potential role of this intracellular enzyme in protecting membrane phospholipids against oxidative stress, platelet-activating factor acetylhydrolase II has been shown to translocate from cytosol to membranes in response to pro-oxidative stressors, and overexpression of this enzyme decreases the cytotoxic effects of these agents. The objective of this study was to assess whether platelet-activating factor acetylhydrolase II is involved in protecting skin against oxidative stress. Platelet-activating factor acetylhydrolase II protein was demonstrated in human skin by immunohistochemistry, with the highest levels of the enzyme found in sebaceous glands and lesser amounts in epidermal keratinocytes. Treatment of epidermal cells with t-butylhydroperoxide or ultraviolet B radiation resulted in platelet-activating factor acetylhydrolase II translocation from cytosol to membranes. To assess the role of this enzyme in epidermal function, a recombinant retroviral strategy was used to overexpress platelet-activating factor acetylhydrolase II in the human keratinocyte-derived cell line HaCaT. Overexpression of platelet-activating factor acetylhydrolase II protected HaCaT cells against apoptosis induced by oxidative stressors t-butylhydroperoxide and ultraviolet B radiation. Similar levels of apoptosis, however, were seen in both control and platelet-activating-factor-acetylhydrolase-II-overexpressing HaCaT cells in response to C2 ceramide. These studies demonstrate the presence of platelet-activating factor acetylhydrolase II in a restricted pattern in human skin, and provide evidence that this specialized phospholipase is involved in protecting this organ against oxidative stress through the degradation of oxidatively modified bioactive phospholipids. ***Key words: apoptosis/keratinocytes/oxidative stress/platelet-activating factor/platelet-activating factor acetylhydrolase. J Invest Dermatol 119:913–919, 2002***

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) is a potent phospholipid-derived mediator implicated in a broad spectrum of physiologic and pathologic activities, particularly inflammation and allergy (reviewed by Pinckard *et al*, 1994; Prescott *et al*, 2000). A variety of human cell types including polymorphonuclear leukocytes, macrophages, endothelial cells, and keratinocytes both synthesize PAF and express functional PAF receptors (PAF-R). The PAF-R is a G-protein-linked transmembrane protein that can signal through many second messenger systems including phospholipases A2, C, and D, as well as by activation of kinases including protein kinase C and mitogen-activated protein kinases (reviewed by Izumi and Shimizu, 1995).

Accumulating evidence suggests that the PAF system is involved in keratinocyte function and skin inflammation. Significant levels of PAF are not found in normal skin, but can be measured in various inflammatory dermatoses ranging from psoriasis to urticaria (Grandel *et al*, 1985; Mallet and Cunningham, 1985). Injection of PAF into skin results in cutaneous inflammation within 1 h, which clinically and histologically resembles an urticarial lesion (Archer *et al*, 1984; Michel *et al*, 1987; Hellewell and Williams, 1989; Travers *et al*, 1998). Human keratinocytes express functional PAF-Rs, which can trigger the production of other pro-inflammatory mediators including eicosanoids, interleukins IL-6, IL-8, and tumor necrosis factor α (Pei *et al*, 1998; Dy *et al*, 1999). Other pro-

Manuscript received July 20, 2000; revised May 15, 2002; accepted for publication May 31, 2002.

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Abbreviations: PAF, platelet-activating factor; PAF-AHII, platelet-activating factor acetylhydrolase II; t-BuOOH, tertiary butyl hydroperoxide.

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oxidative stimuli such as tertiary butyl hydroperoxide (t-BuOOH) and ultraviolet B (UVB) irradiation can also induce the production of PAF or PAF-like species in epidermal cells (Dy *et al*, 1999; Travers, 1999). Of significance, recent studies have implicated PAF in UVB-mediated immunosuppression via epidermal-produced IL-10 (Watersheid *et al*, 2002).

The pathway for the acute synthesis of PAF is the sequential actions of phospholipase A2 and PAF acetyltransferase upon 1-alkyl glycerophosphocholines with a long chain unsaturated fatty acid (often arachidonic acid) at the *sn-2* position. The PAF-R not only recognizes native PAF, but also 1-acyl 2-acetyl glycerophosphocholine analogs as well as *sn-2* short-chained glycerophosphocholines created by free-radical-mediated fragmentation of arachidonic acid or other unsaturated fatty acid (Murphy, 1996; Marathe *et al*, 1999). This production of PAF-like lipids in response to oxidative stress differs from the tightly controlled enzymatic pathway.

PAF is inactivated by removal of the *sn-2* acetate moiety through the actions of a family of specific phospholipases termed platelet-activating factor acetylhydrolases (PAF-AH) (reviewed by Stafforini *et al*, 1997). Unlike phospholipase A2 enzymes, which hydrolyze phospholipids with long chain acyl groups at the *sn-2* position, PAF-AHs selectively hydrolyze short-chained fatty acids at the *sn-2* position of glycerophosphocholines. PAF-AH enzymes can also inactivate short-chained PAF-like lipids produced as a consequence of oxidative stress. In addition to their unusual specificity, PAF-AHs typically are in a fully activated state (Yanoshita *et al*, 1988; Stafforini *et al*, 1991).

Both extracellular or plasma associated and intracellular or tissue associated types of PAF-AHs have been described (Stafforini *et al*, 1997). Extracellular PAF-AH is a 43 kDa monomeric enzyme, which is thought to regulate plasma PAF levels in the plasma. Approximately 4% of the Japanese population have a mutation in PAF-AH abolishing the activity of this enzyme. The presence of this mutation has been reported to be a severity factor for the development of severe asthma (Miwa *et al*, 1998). Two separate intracellular types of PAF-AHs have been described (types I and II). Type I PAF-AH has two isoforms, tentatively named Ia and Ib. Isoform Ib is a heterotrimeric enzyme restricted to brain, the lack of which results in malformation of the cerebral cortex (Miller–Dieker lissencephaly) (Hattori *et al*, 1994).

The intracellular isoform II (PAF-AHII) is a 40 kDa monomer most abundantly expressed in bovine liver and kidney (Hattori *et al*, 1995; 1996). Recent studies using MDBK cells have found that PAF-AHII translocates from cytosol to membranes within 20 min following exposure of the cells to pro-oxidative stressors. Conversely, this enzyme translocates back to cytosol following treatment with antioxidants (Matsuzawa *et al*, 1997). In addition, overexpression of PAF-AHII in Chinese hamster ovary cells inhibited apoptosis induced by the pro-oxidant t-BuOOH (Matsuzawa *et al*, 1997). These findings suggest that this enzyme acts to degrade oxidatively fragmented phospholipids that are toxic to the cell.

The objective of our studies was to assess whether human skin expresses PAF-AHII. Because this enzyme has been hypothesized to protect cells against oxidative stress, we tested whether overexpression of the enzyme in an epidermal cell line could protect against the cytotoxic effects of pro-oxidative stressors including t-BuOOH and UVB.

MATERIALS AND METHODS

Reagents PAF, t-BuOOH, and routine chemicals were obtained from Sigma (St. Louis, MO). Because of its chemical instability, t-BuOOH bottles were discarded within 48 h after opening. Growth media and supplements were purchased from Life Technologies (Gaithersburg, MD) and fetal bovine serum from Intergen (Purchase, NY).

Cells Primary cultures of neonatal-foreskin-derived human keratinocytes and fibroblasts were harvested and cultured as previously outlined (Travers *et al*, 1996). The immortalized sebaceous gland cell line SZ95 was grown in Sebomed medium as described previously (Zouboulis *et al*, 1999).

Generation of monoclonal antibodies against PAF-AHII The cDNA for the PAF-AHII (Hattori *et al*, 1996) was ligated into the NdeI/HindIII sites of the pET21a vector (pET system, Novagen). After the plasmid had been introduced into *Escherichia coli* strain BL21 (DE3), protein was expressed as His-tagged protein by induction with 1 mM isopropyl-b-D-thiogalactopyranoside. The protein was purified using nickel column chromatography (Novagen) according to the manufacturer's protocol. Balb/c mice were immunized with the purified protein and monoclonal antibodies were produced using the PAI myeloma cell line. Hybridoma cell lines were established according to the standard protocol.

Immunofluorescence studies The presence of PAF-AHII protein in skin was assessed by immunofluorescence studies using the monoclonal antibody against human PAF-AHII whose synthesis is described above. Biopsies of human normal skin were collected and immediately frozen in Tissue-Tek, optimal cutting temperature (OCT), obtained from Sakura Finetek Europe BV (Zauterwoude, The Netherlands). The frozen block subsequently was stored at –80°C until used. Six to eight micrometer cryostat sections were obtained and stained with the monoclonal antibody against PAF-AHII. Briefly, the sections were fixed in ethanol for 1 min, washed in ddH_2O for 20–30 s, then washed with phosphate-buffered saline (PBS) for 5 min, and then incubated with anti-PAF-AHII monoclonal antibody diluted at 1:100 in PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature. The sections were washed three times with PBS, and then incubated with a fluorescein-conjugated goat antimouse IgG polyclonal antibody diluted at 1:100 in PBS containing 3% BSA for 1 h at room temperature, in the dark. After the incubation with the conjugated fluorescent antibody the sections were washed three times with PBS and mounted using Fluoromount-G and cover slides. Immunostaining patterns were visualized using an Olympus fluorescent microscope. As a control, extra sections from the same specimen, following all the staining procedures, substituting the PAF-AHII antibody with mouse IgG (Sigma) in the incubation with 3% BSA, were stained at the same time.

HaCaT–PAF-AHII model system The immortalized human keratinocyte derived cell line HaCaT (Boukamp *et al*, 1988) was a gift from Dr. Petra Boukamp (German Cancer Research Center, Heidelberg, Germany) and was cultured as previously reported (Travers *et al*, 1996).

To create HaCaT cells stably overexpressing PAF-AHII, the entire human PAF-AHII cDNA was cloned into the MSCV 2.1 retroviral vector and high titer replication-deficient retrovirus was produced as previously described (Pei *et al*, 1998). HaCaT cells were transduced with MSCV 2.1PAF-AHII or empty MSCV 2.1 retrovirus (as control) using polybrene, and infected cells were screened by using G418 as previously reported (Pei *et al*, 1998). PAF-AHII protein was assessed by immunoblotting using the same monoclonal PAF-AHII antibody used in the immunofluorescence studies. Briefly, confluent 10 cm dishes of HaCaTM PAF-AHII and HaCaTM were washed twice with PBS and lyzed with 1.5 ml of lysis buffer (RIPA) for 30 min, disrupted by sonication in a vessel surrounded by ice for three 30 s periods at 45 s intervals using a sonic dismembrator (Fischer Scientific, Pittsburgh, PA). The cell lysate was centrifuged at 35,000 rpm for 10 min; protein in the resultant supernatant was quantitated using a commercial kit (Bio-Rad), and aliquots were placed in Eppendorf tubes and stored at –80°C. The samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12% acrylamide gel and blotted onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (TBS) containing 5% skim milk overnight at 4°C and then washed three times with TBS containing 2% Tween 20 (T-TBS), incubated with the anti-PAF-AHII monoclonal antibody diluted 1:3000 in TBS containing 5% skim milk for 1 h, and incubated with a horseradish-peroxidase-conjugated goat antimouse antibody diluted 1:3000 in TBS containing 5% skim milk. After washing the membrane four times with T-TBS, peroxidase was detected by an enhanced chemiluminescence method using an ECL Western Blotting Detection Set from Hyperfilm ECL (Amersham Life Science). PAF-AHII distribution in membrane *versus* cytosolic fractions of HaCaT cells was assessed as previously described (Matsuzawa *et al*, 1997).

PAF-AH enzymatic activity PAF-AH enzymatic activity of HaCaT cells was measured as previously described (Narahara and Johnson, 1993). Briefly, $4–6 \times 10^6$ cells were trypsinized, washed with PBS, and pelleted; the supernatant was aspirated and the pellets were frozen (–80°C) until analyzed. The samples were thawed on ice and 500 μl of

cold sucrose (0.25 M) was added to each tube. They were then sonicated in a vessel surrounded by ice for five periods of 10 s at 20 s intervals using an ultrasonic device (Ultrasonic, Planeview, NY), and then centrifuged at 12,000 × *g* at 4°C. The resultant supernatant was removed and assayed for PAF-AH enzymatic activity using 1-O-alkyl-2-[^{3}H]acetate-glycerophosphocholine as previously described (Narahara and Johnson, 1993). Samples were run in triplicate.

Measurement of apoptosis in HaCaT cells HaCaTM or HaCaTM PAF-AHII cells were plated at 8×10^5 cells in 10 cm dishes for 24 h. Following washing with medium, the cells were treated with 200 μM t-BuOOH or 100 μM of C2 ceramide, or irradiated with 400 J per m^2 of UVB (all in regular medium) as previously described (Barber *et al*, 1998). The irradiator was an unfiltered Philips F20T12/UVB source (270–390 nm; containing 2.6% UVC, 43.6% UVB, 53.8% UVA). The intensity of the UV source was routinely measured using an International Light radiometer equipped with a UVB detector. At various time points following treatment, the cells were harvested by collection of supernatant and trypsinization of adherent cells and pelleted. Caspase 3 proteolytic activity in cell lysates was measured using a synthetic fluorogenic substrate (DEVD-AMC, Alexid Biochemicals, San Diego, CA; Hurwitz and Spandau, 2000). Briefly, the cells were suspended in lysis buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 2 mM $MgCl_2$, 1 mM dithiothreitol) for 30 min on ice. Following removal of cellular debris by centrifugation, an aliquot of the cell lysate was added to caspase 3 reaction buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mg per ml bovine serum albumin, and 50 μM CPP32-AMC substrate) and incubated at 37°C for 1 h. Release of the fluorescent AMC moiety was measured using a Hitachi F2000 Spectrophotometer (excitation, 380 nm; emission, 460 nm). The fluorescent intensity was converted to moles of AMC by comparison to the fluorescent intensity of standards of AMC (7-amino-4 methylcoumarin; Molecular Probes, Eugene, OR). The specific activity of caspase 3 in cell lysates was then determined after the total protein concentration of the cell lysates was measured (Nano Orange Protein Quantification Reagent, Molecular Probes) and was expressed as pmoles AMC per h per μg protein.

RESULTS

PAF-AHII expression in human skin Our first studies assessed whether PAF-AHII protein was expressed in human skin. Immunofluorescence studies with a PAF-AHII specific monoclonal antibody revealed expression of this protein in normal human skin. As shown in **Fig 1**, sebaceous glands exhibited the highest levels of PAF-AHII expression with lesser amounts found in the epidermis. Weak PAF-AHII immunoreactivity was expressed throughout the epidermis, yet significant levels of PAF-AHII immunoreactivity were not seen in dermal fibroblasts or blood vessels. Examination of PAF-AHII protein levels in primary cultures of human keratinocytes, fibroblasts, and the immortalized sebaceous gland cell line SZ95 (Zouboulis *et al*, 1999) revealed the highest levels of immunoreactive protein in the sebaceous gland cell line (**Fig 2**). These studies indicate that human skin expresses PAF-AHII protein in a restricted pattern with the highest amounts of protein associated with sebaceous glands/sebocytes.

Translocation of PAF-AHII protein Recent studies have found that PAF-AHII translocates from cytosol to membranes following exposure of the cells to the pro-oxidative stressor t-BuOOH (Matsuzawa *et al*, 1997). The next studies assessed whether the PAF-AHII expressed in epidermal cells translocates in response to oxidative stressors. HaCaT keratincoytes were treated with 100 μM t-BuOOH or irradiated with 600 J per m^2 UVB and the levels of PAF-AHII protein in cytosol *versus* membranes was evaluated. As shown in **Fig 3**, resting HaCaT cells express approximately equal amounts of PAF-AHII protein in membrane *versus* cytosolic fractions. Treatment with UVB or t-BuOOH resulted in increased levels of protein in the membrane over cytosolic fractions.

HaCaT–PAF-AHII model system Through its ability to recognize and inactivate biologically active PAF as well as

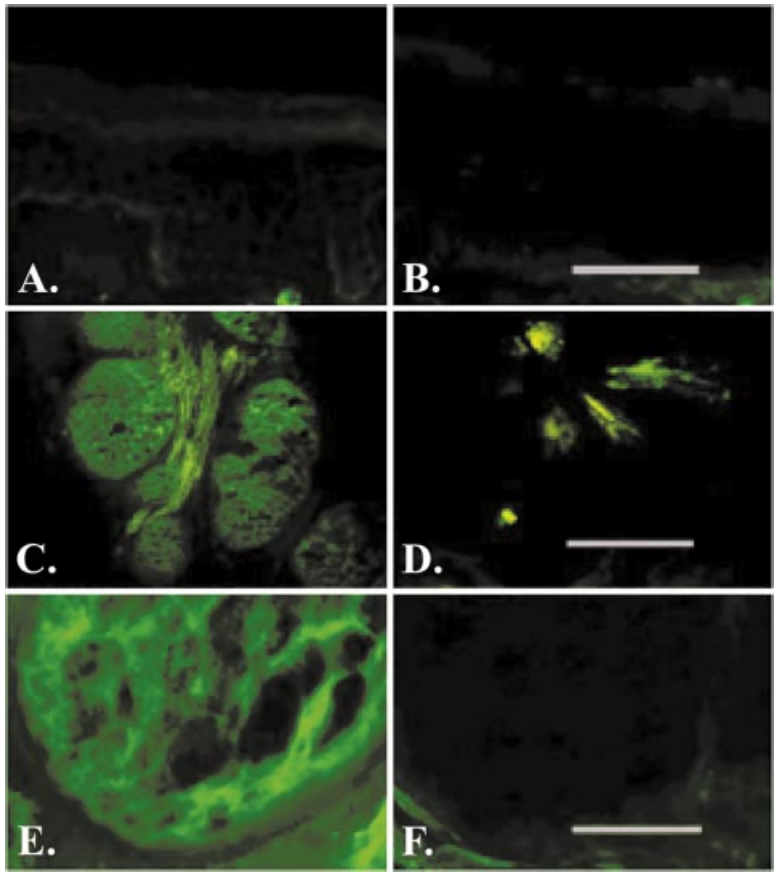

Figure 1. PAF-AHII expression in human skin. Immunofluorescence staining of normal human skin using a monoclonal anti-PAF-AHII antibody. (*A*) Epidermis stained with anti-PAF-AHII antibody (400×). (*B*) Control, using mouse IgG (400×; *bar*: 60 μm). (*C*) Sebaceous glands stained with anti-PAF-AHII antibody (100×). (*D*) Control (100×; *bar*: 240 μm). (*E*) High power of sebaceous glands stained with anti-PAF-AHII antibody (400×). (*F*) Control (400×; *bar*: 60 μm).

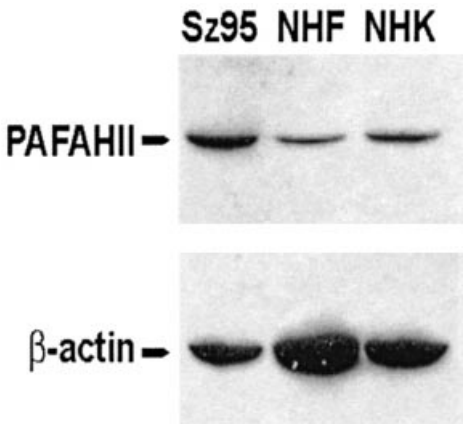

Figure 2. Measurement of PAF-AHII protein in keratinocytes, fibroblasts, and a sebaceous cell line. Protein (40 μg) was isolated from primary cultures of neonatal-foreskin-derived human keratinocytes (NHK), human fibroblasts (NHF), and SZ95 sebocytes, and run on a 15% sodium dodecyl sulfate polyacrylamide gel. PAF-AHII immunoreactivity was determined using a monoclonal antibody. The membranes were stripped and re-probed with an antibody against β-actin.

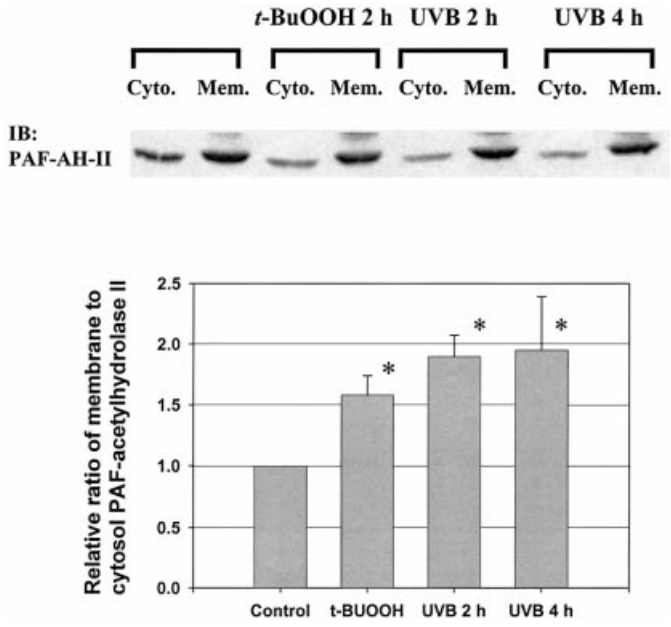

Figure 3. Effect of t-BuOOH and UVB on distribution of PAF-AHII levels in HaCaT keratinocytes. (*A*) HaCaT cells were treated with 100 μM t-BuOOH for 2 h or were exposed to 600 J per m^2 UVB. Two or 4 h after UVB treatment the cellular cytosol and membrane fractions were isolated by ultracentrifugation and run on a 15% sodium dodecyl sulfate polyacrylamide gel; PAF-AHII immunoreactivity was determined using a monoclonal antibody. (*B*) Immunoblots were scanned and quantified using NIH Image software. Each value is the relative ratio and standard deviation of membrane to cytosol PAF-AHII obtained from three independent experiments and normalized to the untreated ratio. An asterisk represents a significant difference between translocation of PAF-AHII in untreated and treated cells using analysis of variance with Neuman–Keuls as the *post hoc* test ($p < 0.05$).

PAF-like lipids created in response to oxidative stress, PAF-AHII is thought to play a protective role in the skin. Consistent with the notion that PAF-AHII could protect cells against oxidative stress, overexpression of this protein in Chinese hamster ovary cells inhibits t-BuOOH-induced apoptosis (Matsuzawa *et al*, 1997). As

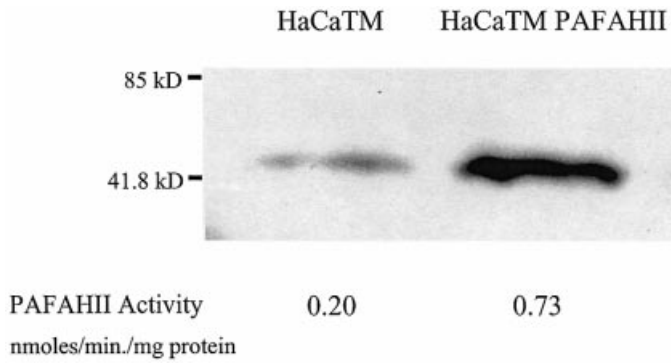

Figure 4. Measurement of PAF-AHII protein and enzyme activity in HaCaT cells. Protein (10 μg) was extracted from HaCaTM PAF-AHII and control HaCaTM cells and immunoblotted using a monoclonal PAF-AHII antibody. PAF-AH enzyme activities of cells are listed below the corresponding immunoblot, and are the mean of three separate samples. The standard deviation of the enzyme activity measurements was less than 10%.

immunofluorescence studies suggest that human keratinocytes express PAF-AHII, we created a model system using the human keratinocyte-derived cell line HaCaT, which was genetically engineered to overexpress PAF-AHII using a replication-deficient retrovirus. Northern blotting of HaCaT cells transduced with PAF-AHII (HaCaTM PAF-AHII) or control transduced (HaCaTM) demonstrated that both cell populations contained PAF-AHII mRNA, with less than a 2-fold increase found in the HaCaTM PAF-AHII cells (data not shown).

Immunoblotting studies revealed increased levels of PAF-AHII protein in HaCaTM PAF-AHII over HaCaTM cells. Enzyme activity studies demonstrated a greater than 3-fold increase in PAF-AH activity in the HaCaTM PAF-AHII over HaCaTM cells (**Fig 4**). Using this model system, the role of moderate over-expression of PAF-AHII in a keratinocyte-derived cell line could be assessed.

Protective effects of PAF-AHII overexpression on apoptosis induced by t-BuOOH and UVB irradiation The next experiments investigated whether overexpression of PAF-AHII in HaCaT cells protects against apoptosis induced by oxidative stressors such as t-BuOOH and UVB irradiation. Apoptosis was assessed by measurement of caspase 3 enzymatic activity using a fluorescent substrate as outlined in *Materials and Methods*. Our laboratory has previously demonstrated that caspase 3 enzymatic levels correlate with other apoptotic measurements in epithelial cells (Southall *et al*, 2001). HaCaTM and HaCaTM PAF-AHII cells were treated with 100 μM t-BuOOH and incubated at 37°C, and caspase 3 enzymatic activity was assessed at various times. As shown in **Fig 5**, t-BuOOH treatment resulted in increased caspase 3 specific activity by 8 h in HaCaTM cells. Maximal levels of caspase 3 induction were seen by 12 h. HaCaTM PAF-AHII cells did not exhibit increased caspase 3 enzymatic activity until 24 h, however. Consistent with the cytotoxic effects described in other cell types, HaCaTM cells treated with t-BuOOH began to detach from the plate by 8 h. In contrast, the majority of HaCaTM PAF-AHII cells remained attached to the plate and viable until 24 h. These studies indicate that overexpression of PAF-AHII protein is protective against cellular cytotoxicity induced by the oxidative stressor t-BuOOH.

Recent studies suggest that UVB acts as a potent pro-oxidative stimulus in human keratinocytes (Peus *et al*, 1998). As our previous studies indicate that PAF and PAF-like lipids are produced by epidermal cells in response to UVB (Barber *et al*, 1998; Dy *et al*, 1999), we tested whether overexpression of PAF-AHII could protect against UVB-induced apoptosis. Irradiation of HaCaTM and HaCaTM PAF-AHII cells with 400 J per m^2 UVB resulted in

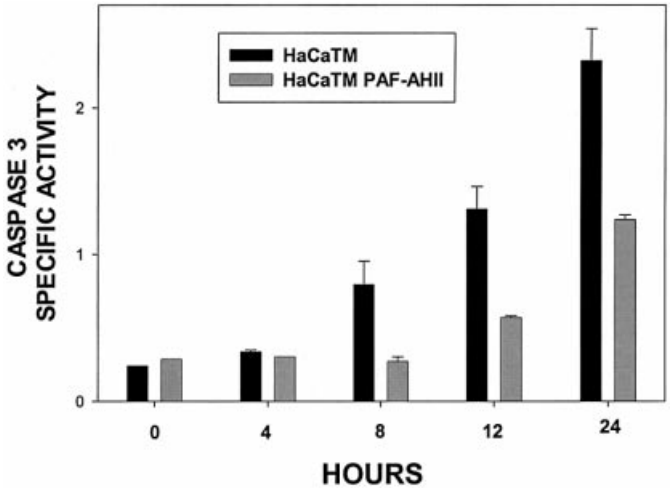

Figure 5. t-BuOOH-induced apoptosis in HaCaTM and HaCaTM PAF-AHII cells. HaCaTM and HaCaTM PAF-AHII cells were treated with 100 μM t-BuOOH and incubated at 37°C. The cells were harvested at time points as indicated. Caspase 3 specific enzymatic activity was then assessed. Each point represents the mean ± SD of caspase 3 specific activity from a typical experiment from three separate experiments with similar results.

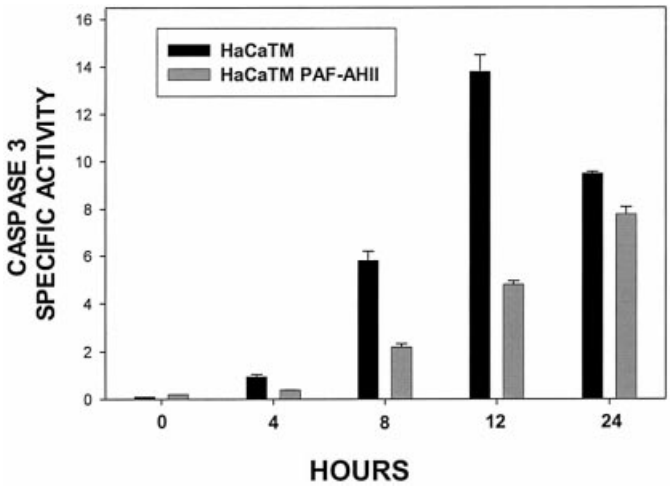

Figure 6. UVB-radiation-induced apoptosis in HaCaTM and HaCaTM PAF-AHII cells. HaCaTM and HaCaTM PAF-AHII cells were irradiated with 400 J per m^2 and incubated at 37°C. The cells were harvested at time points as indicated. Caspase 3 specific enzymatic activity was then assessed. Each point represents the mean ± SD percentage of the peak of caspase 3 specific activity from a typical experiment from three separate experiments with similar results.

caspase 3 activation (**Fig 6**). The levels of caspase 3 were significantly less in HaCaTM PAF-AHII *versus* HaCaTM cells at 8 and 12 h, however. Examination of the plates for detached cells mirrored the caspase 3 enzymatic assays, with fewer adherent cells in HaCaTM *versus* HaCaTM PAF-AHII cells until 24 h. These findings indicate that increased levels of PAF-AHII protein delays UVB-induced apoptosis.

Lack of protective effects of PAF-AHII overexpression on apoptosis induced by C2 ceramide C2 ceramide is a short-chained ceramide that induces apoptosis in many cell types (Obeid and Hannun, 1995). Although C2 ceramide has been reported to induce oxidative stress in mitochondria (Ballon et el, 1996), this toxic lipid does not have the pro-oxidative effects of t-BuOOH or UVB. Treatment of HaCaT cells with 100 μM C2 ceramide resulted in apoptosis by 24 h as measured by enhanced caspase 3

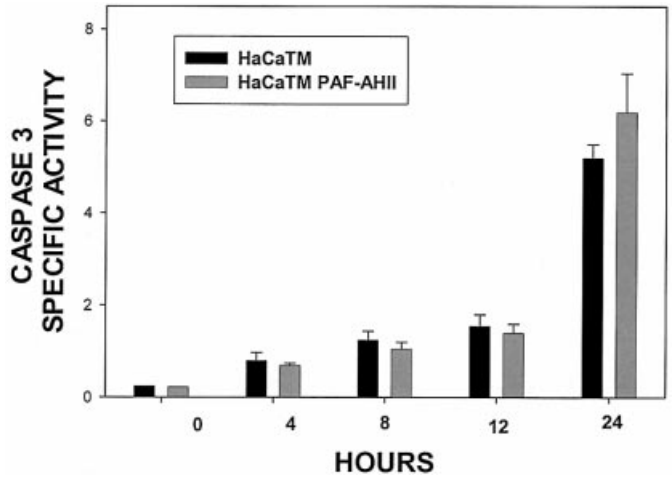

Figure 7. C2-ceramide-induced apoptosis in HaCaTM and HaCaTM PAF-AHII cells. HaCaTM and HaCaTM PAF-AHII cells were treated with 100 μM of C2 ceramide and incubated at 37°C. The cells were harvested at time points as indicated. Caspase 3 specific enzymatic activity was then assessed. Each point represents the mean ± SD percentage of the peak of caspase 3 specific activity from a typical experiment from three separate experiments with similar results.

induction (**Fig 7**). The amount of caspase 3 induction as well as numbers of detached cells were similar in HaCaTM *versus* HaCaTM PAF-AHII cells, however, suggesting that overexpression of PAF-AHII does not protect against C2-ceramide-induced apoptosis.

DISCUSSION

Keratinocytes are chronically exposed to a powerful oxidant agent UVB, as well as endogenous sources of reactive oxygen species from metabolic processes and also from inflammatory cells such as neutrophils. As both oxidized lipids and the PAF system appear to be involved in epidermal function/cutaneous inflammation, characterization of the enzymes involved in the regulation of these lipids is important. These studies demonstrate that human skin contains the specialized phospholipase PAF-AHII, and provide evidence that this enzyme could be involved in the protection of this organ against the toxic effects of oxidative stress.

Accumulating evidence indicates that unsaturated fatty acids are a target for oxidative stress. Indeed, reactive oxygen species have been shown to spontaneously oxidize unsaturated fatty acyl chains in membrane phospholipids (Zimmerman *et al*, 1995). Depending upon the structure of the resultant product, oxidized lipids can have biologic activities through activation of the PAF-R, as well as cause cell damage and apoptosis through receptor-independent effects. Thus, oxidized phospholipids need to be rapidly hydrolyzed to prevent their cellular toxicities.

PAF-AHII is a recently cloned protein whose enzymatic activity is that of a phospholipase A2, yet restricted to the hydrolysis of short-chained *sn-2* acyl groups. Several features of this enzyme suggest that it could be involved in protecting cells against oxidized phospholipids. First, PAF-AHII is constitutively active, unlike other phospholipase A2 enzymes (Blank *et al*, 1981; Stremler *et al*, 1989; Tjoelker *et al*, 1995). Second, studies using MDBK renal cells and now HaCaT keratinocytes indicate that PAF-AHII translocates from cytosol to membranes following exposure to oxidants. The ability of this active enzyme to rapidly translocate to membranes would ensure that oxidized unsaturated acyl groups in the *sn-2* position of phospholipids are rapidly degraded. Finally, over-expression of PAF-AHII in Chinese hamster ovary cells protects against t-BuOOH-induced apoptosis (Matsuzawa *et al*, 1997). Our studies are also consistent with the notion that this enzyme is involved in protecting cells against oxidized phospholipids.

In this paper we present evidence that PAF-AHII is expressed in a restricted pattern in human skin. Immunofluorescence, northern blotting, and enzymatic assays demonstrate that PAF-AHII is expressed in keratinocytes. The highest levels of PAF-AHII expression are found in sebaceous glands, however, including the sebaceous gland cell line SZ95, shown to exhibit major characteristics of human sebocytes *in vitro* (Zouboulis *et al*, 1999). This higher level of PAF-AHII expression in sebaceous glands, which have a high lipid content, fits with an increased need of this tissue to protect itself against oxidatively modified phospholipids. It may also indicate a protective action of the sebaceous glands on other neighboring skin structures against oxidative stress via a paracrine pathway.

To evaluate potential protective effects of PAF-AHII on pro-oxidative stressors in keratinocytes, a replication-deficient retrovirus was used to stably overexpress this enzyme in HaCaT cells. As shown in **Fig 4**, HaCaTM PAF-AHII cells had an approximately 300% increase in baseline PAF-AH enzymatic activity over baseline HaCaTM cells. This moderate overexpression of PAF-AHII resulted in protection against the cytotoxic effects of t-BuOOH and UVB irradiation. The protection was manifested as a delay in the onset of apoptosis induced by these oxidative stressors. That PAF-AHII did not fully protect against the cytotoxic effects of these agents is compatible with the notion that other targets for UVB and t-BuOOH that are independent of oxidized phospholipids also play an important role. Increased levels of PAF-AHII, however, did not affect apoptosis induced by C2 ceramide, a nonoxidative stressor. These findings fit with previous studies where overexpression of PAF-AHII was found to be protective against t-BuOOH-induced apoptosis (Matsuzawa *et al*, 1997). Although all these studies point to a protective effect of overexpression of this enzyme on oxidative stress, we have found that high levels of overexpression (4000%–8000%) in HaCaT or KB cells using a mammalian expression vector result in increased levels of both spontaneous and t-BuOOH-induced apoptosis (data not shown). These findings might be explained by recent studies demonstrating that PAF-AHII has the ability to transfer the short chain *sn-2* group to sphingosine, producing short-chained ceramides including C2 ceramide, which has known cytotoxic effects (Karasawa *et al*, 1999). Thus, this enzyme not only can inactivate PAF and related short-chained *sn-2* phopsholipids, but can generate other diverse lipid mediators.

Both oxidized lipids and the PAF system appear to have the potential to modulate cutaneous inflammation as well as to have cytotoxic effects. Our studies demonstrate that PAF-AHII, a novel phospholipase that serves to regulate levels of both, is found in human skin. Moderate overexpression of PAF-AHII in the human keratinocyte cell line HaCaT protects against oxidative stressors *in vitro*, suggesting that this enzyme could be involved in protecting skin from oxidative damage *in vivo*. In addition, this enzyme has anti-inflammatory effects through its ability to degrade PAF. Though genetic mutations with resultant clinical effects have been described for other forms of PAF-AHs in humans, it is not known whether PAF-AHII mutations are associated with disease. Future studies are planned to examine PAF-AHII levels in human skin diseases that are associated with exaggerated responsiveness to oxidative stress, including photosensitivity disorders.

The authors wish to acknowledge the technical assistance of Drs Silvio Marques and Dan Spandau (I.U. Department Dermatology). These studies are supported in part by grants from the Showalter Memorial Fundation, the Riley Memorial Association, and the National Institutes of Health grants AR1993 and HL62996. M.M. was supported by Brazilian grants from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo- FAPESP. M.S. was supported by a grant from the Dermatology Foundation. H.S. was supported by a grant from the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV Z 5.1-1328-156).

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